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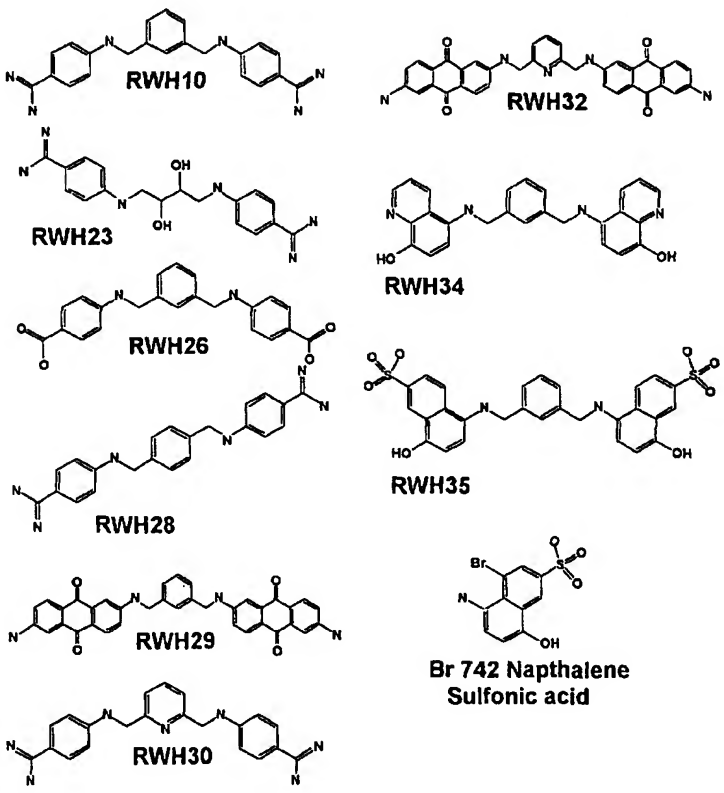

(57) Abstract: The present invention is a series of novel and effective inhibitors of integrase, an essential in the life cycle of retroviruses. These compounds were designed to have a restricted conformation for the determination of the integrase binding site and mechanism of inhibition. The integrase inhibitors of the present invention are effective in the submicromolar range, and thereby provide novel lead compounds for the development of anti-viral therapeutics.

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SYMMETRIC INHIBITORS OF HIV INTEGRASE, MAMMALIAN TOPOISOMERASE AND SERINE PROTEASE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119 based upon U.S. Provisional Application No.60/215,474 filed June 30, 2000.

FIELD OF THE INVENTION

The present invention relates to the field of biochemistry, and more particularly to the design of symmetric compounds with restricted conformation space, thereby allowing for the examination of the HIV integrase binding site and, subsequently, the mechanism of inhibition of enzyme activity.

BACKGROUND OF THE INVENTION

Acquired immune deficiency syndrome, more commonly known as AIDS, is caused by a retrovirus, a human immunodeficiency virus (HIV), HIV-1. The genome of HIV is composed of RNA consisting of a *gag* sequence, which encodes the viral core proteins; *pol,* which encodes the viral enzymes; and *env*, which encodes the envelope glycoproteins. Following viral adsorption to the target cells, which are most commonly T-lymphocytes, the viral reverse transcriptase creates a double-stranded DNA copy of the viral genomic RNA. Integration of this retroviral DNA into the host cell chromosome is a critical step in the life cycle of the retrovirus. All retroviruses encode an integrase (IN) that performs this essential activity (Asante-Appiah, E., and Skalka, A.M *Antiviral Res.* 36:

139-152, 1997). The retroviral integrase is encoded in the 3' end of the *pol* gene and is assembled into the viral particle as part of the Gag-Pol precursor. Since the integrase is essential for viral replication, and no cellular counterpart has been found, the virus-encoded integrase is an attractive target for selective inhibition of the AIDS virus (Nicklaus, M.C., et al., *J. Med. Chem.*40:920-929, 1997).

Integrases from avian sarcoma-leukosis virus (ASLV) and human immunodeficiency virus (HIV) have been studied extensively. Following reverse transcription of the viral RNA into a double-stranded DNA, the retroviral DNA becomes associated with the integrase. The enzyme reaction is site-specific for the viral DNA and can be divided into two chemical steps. First, the viral DNA processing step removes (usually) two nucleotides from each each strand of the 3'end of the retroviral DNA, resulting in recessed 3'-OH termini. This is followed by a joining step involving a transesterification reaction in which a host DNA strand is cut and the 5' end of the cut is joined to a processed 3' end of the viral DNA (Pommier, Y., et al., *Antiviral Chem & Chemotherapy* 8(6): 463-483, 1997; Fesen, M.R., et al., *Biochem Pharm* 48: 595-608, 1994). Joining to the host cell DNA appears random *in vivo*, although preferred target DNA structures have been detected *in vitro* (Katz, R. A., et al., *Virology* 217: 178-190, 1996). This integration of the retroviral genome into the host cell genome is followed by the synthesis of viral proteins and RNA, with the subsequent production of infectious virions.

The structure of the isolated catalytic core domain of ASLV integrase has been determined by x-ray crystallography (Bujacz et al, *J. Mol. Biol*, 253:333-346, 1995; Wlodawer, A. and Vondrasek, J., *Annu. Rev. Biophys. Biomol. Struct.* 27,249-284, 1999), and the structures of each of the three separated domains of HIV-1 integrase have been determined by crystallography or NMR (reviewed in Esposito & Craigie, *Adv. Virus Res.*, 52, 319-333, 1999). The central, core domain is responsible for the enzymatic activity of integrase, and its structure resembles other

nucleases and polynucleotidyl transferases, such as RNase H and MuA transposase. The small N-terminal domain of HIV-1 integrase is required for integration activity, and the C-terminal domain for DNA binding and oligomerization (Asante-Appiah, E., and Skalka, A.M *Antiviral Res.* 36: 139-152, 1997). Since integration of the viral genome into the host chromosomal DNA is a prerequisite for the production of infectious virus particles, an active integrase is an essential component of the virus, and thus a desirable target for anti-viral therapy.

HIV integrase is a potential target for antiviral therapy because it provides an essential function in viral replication. Inhibitors of the other two HIV-1 enzymes, administered in combinations, are currently the major source of anti-retroviral therapy for AIDS. Inhibitors of reverse transcriptase block the early step of formation of viral DNA from RNA, and inhibitors of the viral protease which is required for proteolytic maturation of the viral particle, block at the other end of the viral life cycle. Because the virus develops resistance to drugs rapidly, additional targets for therapy are desirable. Improved anti-HIV activity was shown by the combination of an integrase inhibitor with reverse transcriptase and protease inhibitors (Robinson W.E., *Antiviral Res.* 39,101-111, 1998). However, effective specific inhibitors of the HIV integrase are not currently available. Although limited by the lack of a structure of the full length protein or an integrase-DNA complex, the development of new inhibitors of this enzyme is none-the-less an important area of current research (King et al., *J. Med. Chem.* 42, 497-509, 1999; Mekouar et al., *J. Med. Chem.* 41, 2846-2857, 1998; Zhao et al., *J. Med. Chem.*, 40, 242-249, 1997). Recently, the crystal structure of an inhibitor complex with the catalytic core of HIV integrase was determined (Goldgur et al., *Proc. Natl. Acad. Sci. U.S.A* 96, 13040-13043, 1999), and effective anti-viral integrase inhibitors were reported (Hazuda et. al., *Science* 287:646-650, 2000).

The compounds of the present invention have similar IC_{50} values to the Merck compounds L-731,988 and L-708,906 (Hazuda et. al., *Science*,

287:646-650, 2000), both in *in-vitro* and in cell culture assays. Unlike the Merck compounds, which were discovered by screening more than 250,000 samples, the compounds of the present invention were developed by rational methods, starting from a pentamidine analog. A total of nine compounds were synthesized and tested to result in a bioactive sub-micromolar inhibitor. The *in vitro* assay used for this work measured the 3'-end processing step, showing that inhibition of strand transfer is not a necessary prerequisite for inhibition of viral integration in cell culture. The compounds of the present invention, unlike both the Merck compounds and the previously reported 5CITEP (Goldgur et al., *Proc. Natl. Acad. Sci. U.S.A* 96, 13040-13043, 1999), are not di-ketones, but are bi-functional aromatics which are believed to function as nucleotide analogs.

Rational drug design has contributed to the development of the clinically useful inhibitors of HIV protease (Wlodawer and Vondrasek, *Annu. Rev. Biophys. Biomol. Struct.* 27, 249-284, 1998). The novel series of compounds described in the present invention were developed as an outgrowth of work in rational design of inhibitors of a different enzyme (Kurinov & Harrison, *Nature Structural Biology,* 1(10) 735-743, 1994). These compounds, related to pentamidine, were developed initially as serine protease inhibitors to study the roles of these enzymes in several biological processes. However, pentamidine, and many pentamidine derivatives, also inhibit topoisomerases (Boykin, et. al., *J. Med. Chem.* 38, 912-916, 1995).

The present invention tests a series of 9 newly synthesized compounds and pentamidine for inhibition of purified HIV-1 integrase (residues 1-288), ASLV integrase (residues 1-286), and the ASLV integrase catalytic core (residues 52-207). The best inhibitors in this series, RWH34 and 35, had IC_{50} values of about 0.7 μM for HIV-1 integrase and 6 μM for ASLV integrase under the conditions tested. In addition, during the synthesis of RWH35 a side reaction resulted in the generation of 8-bromo,

7-amino, 4-hydroxy, 2 naphthalene sulfonic acid. All data pertaining to RWH35, therefore, also pertains to 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid. It is noted that RWH35 is used alone, 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid is used alone, and a mixture of RWH35 and 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid is used as an inhibitor of integrases. Activity against both proteins implies that the inhibitors target a conserved region of the integrase. By use of a fluorescence assay, RWH35 was shown to bind directly to both proteins in the absence of DNA. The inhibitors were shown to be reversible inhibitors by order of addition experiments. The binding of the inhibitors to the catalytic core of HIV integrase was modeled by analogy to the crystal structure of Goldgur (Goldgur et al., *Proc. Natl. Acad. Sci. U.S.A* 96, 13040-13043, 1999). Finally, RWH35 was shown to be effective in blocking viral integration in cell culture. Therefore, the integrase inhibitor RWH35, has the characteristics of a potential lead compound for development of clinical anti-retroviral agents.

SUMMARY OF THE INVENTION

The present invention relates to integrase inhibiting compounds that contain a linker group, at least one functional group replacing an atom on the linker group and a flexible linkage bond between the functional group and the linker group.

The linker group on the inhibitor compound of the present invention is a rigid aromatic compound or a rigid non-aromatic compound. In one embodiment of the present invention the rigid aromatic compound is a planar rigid aromatic compound. In one embodiment of the present invention the linker group of an inhibitor containing a rigid aromatic compound is a *meta*-xylene. In one embodiment of the present invention the linker group of an inhibitor containing a rigid aromatic compound is a

para-xylene. In one embodiment of the present invention the linker group of an inhibitor containing a rigid aromatic compound is a 2,6- dimethyl pyridine. In another embodiment of the present invention the linker group of an inhibitor containing a rigid non-aromatic compound is 1,4-diamino 2,3-butanediol.

The functional group of the compounds of the present invention is at least one derivitized mono or poly cyclic aromatic group. In one embodiment of the present invention the functional group on inhibitors that contain a rigid aromatic or a rigid non-aromatic compound is a *para*-amino benzamidine. In one embodiment of the present invention the functional group on inhibitors that contain a rigid aromatic or a rigid non-aromatic compound is a; *para*-amino benzoic acid. In one embodiment of the present invention the functional group on inhibitors that contain a rigid aromatic or a rigid non-aromatic compound is a 2,6 diaminoanthraquinone. In one embodiment of the present invention the functional group on inhibitors that contain a rigid aromatic or a rigid non-aromatic compound is a 5-amino, 8-hydroxy quinoline. In one embodiment of the present invention the functional group on inhibitors that contain a rigid aromatic or a rigid non-aromatic compound is a 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid.

One embodiment of the present invention is a symmetric inhibitor. Another embodiment of the present invention is an asymmetric inhibitor.

It is another object of the present invention for the functional group on inhibitors that contain a rigid aromatic compound to mimic nucleotides or bases of DNA. It is a further object of the present invention for these nucleotides or bases to be placed in a stable hydrogen bonding geometry and base stacking geometry on the integrase.

It is a further object of the invention to present a method of inhibiting an integrase whereby an integrase inhibiting compound binds to an intergrase. The integrase inhibiting compound has a rigid aromatic linker group and at least one functional group replacing an atom of the rigid aromatic linker group. The functional group is bonded to the linker

group by a flexible linkage. The functional group is a derivitized mono or poly cyclic aromatic group. The integrase inhibiting compound binds to the integrase and inhibits a catalytic activity of the integrase, thereby inhibiting an essential function in viral replication of the retrovirus.

In one embodiment of the present invention the method of inhibiting the integrase is by the integrase inhibiting compound binding at or close to the active site on the integrase. In another embodiment of the present invention the method of inhibiting the integrase is by the integrase inhibiting compound binding in a site distal to or distinct from the DNA binding site on the integrase.

In one embodiment of the present invention the method of inhibiting the integrase is by the integrase inhibiting compound binding directly to a viral DNA-integrase complex. In another embodiment the method of inhibiting the integrase by the integrase inhibiting compound is by a direct binding to the viral and host DNA-integrase complex. In another embodiment of the present invention the integrase inhibiting compound inhibits the integrase by inhibiting viral integration of the viral DNA into the host DNA. In a further embodiment of the present invention the method of inhibiting an integrase is by inhibiting a processing step of the integration of the viral DNA into the host DNA.

The present invention further relates to an integrase inhibiting compound that contains a rigid aromatic linker group, at least one functional group replacing an atom on the linker group and a flexible linkage bond between the functional group and the linker group, whereby the integrase inhibiting compound is a lead compound for further development of a therapeutic agent that causes inhibition of an integrase. The functional group on this compound isa derivitized mono or poly cyclic aromatic group. In one embodiment of the present invention the inhibition of an integrase by a therapeutic agent is by the inhibition of the replication of a virus at concentrations that are below cell toxicity.

The present invention further relates to an integrase inhibiting compound that is a mixture of 8-bromo, 7-amino, 4-hydroxy, 2

naphthalene sulfonic acid and a diamino methyl meta-xylene linker group with two functional groups, the functional groups being 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid. In another embodiment an integrase inhibiting compound is 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid.

ABBREVIATIONS:

"Kapp" means "apparent dissociation constant"

"IN" means "integrase"

DEFINITIONS:

RWH35 means

a diamino methyl meta-xylene linker group with two functional groups, the functional groups being 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid, or 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid, or a mixture of a diamino methyl meta-xylene linker group with two functional groups, the functional groups being 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid, and 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid

DESCRIPTION OF THE DRAWINGS

Figure 1: Structures of compounds tested for inhibition of HIV-1 integrase, ASLV integrase and ASLV integrase catalytic core.

Figure 2: Fluorescence changes with HIV, ASLV and ASLV-core integrase.

A. Inverse plot for estimating the binding constants.

B: Fluorescence changes for RWH35 titration of HIV-1 integrase

Figure 3. Inhibitor kinetics with ASLV (top) and HIV (bottom) integrases for

A. RWH34

B. RWH35

C. order of addition experiment for RWH34 and RWH35 with HIV integrase

Figure 4. Inhibitor RWH35 blocks integration of retroviral DNA after infection of cultured HeLa cells. (A) Inhibitor effects on HIV DNA integration as determined by expression of β-galactosidase expressed from the lacZ reporter gene encoded in the viral vector (Naldini, L., et. al., *Science* 272, 263-267, 1996). Cells were stained and counted 2 days after infection. (B) Inhibitor effects on ASLV DNA integration as determined by expression of a drug resistance (neo^r)reporter gene from an amphoteropic viral vector (Daniel, R., et. al., *Science* 284, 644-647,1999). Drug (G418) resistant colonies were counted 6 days after infection. (C) The effect of inhibitor on the growth of uninfected HeLa cells.

DESCRIPTION OF THE INVENTION

A good strategy to treat resistant HIV is to target a viral function other than reverse transcriptase or protease. Rational drug design of enzyme inhibitors has contributed to the development of viable inhibitors of HIV protease and reverse transcriptase. A similar approach to drug design was used in the present invention, which describes a novel series of

compounds that inhibit integrases (Kurinov, I.V. and Harrison, R.W., *Nature Structural Biology* 1(10): 735-743, 1994). HIV integrase is a good candidate for inhibition, and drugs that inhibit integrase are not yet available. Therefore, the present invention relates to the development of novel symmetric compounds that inhibit the integrases from human immunodeficiency virus (HIV) and avian sarcoma-leukosis virus (ASLV).

Pentamidine and many pentamidine derivatives inhibit topoisomerases (Boykin D.W., et. al, *J. Med. Chem.* 38: 912-916. 1995; Edwards et. al., *Biochemistry* 31: 7104-7109, 1992). Topoisomerases and integrases catalyze similar reactions, in that topoisomerase catalyzes strand exchange to replace a strand of DNA with itself after unwinding and integrase catalyzes strand exchange between two DNA molecules to form one DNA molecule. Since the pharmacology of topoisomerase inhibitors is well developed, while that of integrase inhibitors is not, compounds that are effective against topoisomerases were tested for their efficacy against retroviral integrases.

Pentamidine analogs, like RWH10, were originally developed as serine protease (such as tryptase) inhibitors. Additionally, pentamidine analogs are known topoisomerase inhibitors (*supra*). Due to the similarity in the catalytic reactions of topoisomerases and integrases, the pentamidine analogs were screened for their ability to inhibit retroviral integrase. The initial compounds related to pentamidine were developed to study the roles of various serine proteases in several biological processes. The series of compounds (**Figure 1A-D and Table I**) and pentamidine were assayed for inhibition of avian sarcoma-leukosis virus (ASLV) integrase (residues 1-286), its catalytic core (residues 52-207), and human immunodeficiency virus-1 (HIV-1) integrase (residues 1-288).

Table I: IC_{50} values (μM)

Compound	Hiv-1 integrase -2	ASLV integrase -2	ASLV core -3
RWH10	12	270	410
RWH23	32	390	760
RWH26	50	840	>1000
RWH28	40	>1000	810
RWH29	26	210	110
RWH30	12	140	520
RWH32	18	100	140
RWH34	0.7	6.3	4.1
RWH35	0.8	8.9	13
pentamidine		>1000	>1000

The best inhibitors, RWH34 and 35, had IC_{50} values of about 0.8 μM for HIV IN, and 6 μM for ASLV IN. In preliminary studies, infected cells show 50% reduction in viral DNA integration with ~3 μM inhibitor and no cytotoxicity. Many integrase inhibitors act by binding to the DNA. A fluorescence assay (**Figure 2**) confirmed that the direct binding of RWH35 to the integrase, rather than its binding to the DNA-integrase complex, is the mechanism of inhibition. The efficacy of RWH35, and its affinity for the integrase, reveals that these compounds inhibit the processing step of integration. This inhibitor is a promising lead compound for further development. Comparative *in vitro* studies showed that the same compound inhibited the ASLV full-length integrase and its catalytic core with IC_{50} values of 9 and 13 μM, respectively. However, RWH35 showed a higher affinity for the full-length integrase with Kapp of 14 nM, compared to 120 nM for the core enzyme, suggesting that the N- and C-terminal domains contribute to inhibitor binding.

The mode of inhibition is determined by modeling, crystallography, binding assays with integrase and/or DNA substrate, inhibition of the integration reaction *in vitro* and in infected cells. While there are

currently no crystal structures of the intact integrase or the integrase-DNA complex, models can be built using the crystal structures of the catalytic core and NMR structures of the termini (Esposito & Craigie, *Adv. Virus Res.* 52, 319-333, 1999). The inhibitors are compared to models of the ends of the DNA substrate. The known crystal structures of ASLV and HIV core-inhibitor complexes allowed for the modeling of the interaction of the new inhibitors to the integrase core to be analyzed. Such modeling revealed that the new inhibitors would dock to the integrase core (Lubkowski, J., et al., *Proc Natl Acad Sci U S A* ,95: 4831, 1998; Goldgur et al., *Proc. Natl. Acad. Sci. U.S.A* 96, 13040-13043, 1999).

The compounds are assayed for inhibition of the integration reaction and inhibition of the virus's ability to infect cells. In addition, crystallization of the integrases with the new inhibitors is tested. Although the compounds of the present invention inhibit cellular proliferation, the concentrations of drug required to achieve this effect are 4-10 fold greater then those required to inhibit the viral infectivity of cells.

Modifications of the best inhibitor are designed based on structural models of both preprocessed DNA and the integrase. The current hypothesis is that these inhibitors mimic the end of a DNA duplex molecule or a DNA duplex with a single strand. Altering the compounds to explore the binding site tests this hypothesis. Systematic variation of the linker and functional groups, which are bonded to each other with a flexible linkage, led to the current effective compounds and these compounds are pursued further to study the structural determinants of inhibition. The functional groups are derivitized aromatic phenyl naphthyl groups. One group of inhibitors contains functional groups that mimic adenine and guanine bases. These functional groups are changed to mimic other bases in addition to adenine and guanine. Further, compounds that place functional groups into a stable hydrogen bonding geometry and base stacking geometry on the integrase are included in the present invention. Although rigid aromatic linker chemistry has proven to

be the best in the current set of designs, rigid non-aromatic linkers are also tested, as well as linkers which alter the locations of the flexible links in the inhibitors.

The inhibitors of the present invention are symmetric, primarily for reasons of synthetic convenience. Alternate chemistry is also explored to generate asymmetric compounds. Current compounds are synthesized in one or two steps to high purity and assayed for binding to the integrase using tryptophan fluorescence. Comparative assays on HIV and ASLV integrases will aid in designing more specific inhibitors and in analysis of the mechanism of inhibition.

Methods

Synthesis of Compounds

Compounds were synthesized by alkyl-halide substitution on an aryl-amine. A bi-alkyl-halide, such as αα'dibromomethyl *meta*-xylene, was reacted with two equivalents of an aryl amine, such as *para*-amino benzamidine, by dissolving the reactants in dimethylsulfoxide (DMSO) and heating. Unlike alkyl-amines where control of the substitution is tricky, aryl amines are less reactive and sterically blocked resulting in effective control of the reaction. Reactions were monitored with thin layer chromotography (TLC), using either 2:1(v:v) butanol:NH_4OH(aqueous, saturated) or 7:3(v:v) hexane:ethylacetate on silica gel. The butanol:ammonia was usually sufficient to resolve product from reactants, but the hexane:ethylacetate was useful for monitoring the alkyl halide. DMSO solutions were either dried and resuspended in methanol or diluted with methanol to avoid artifacts due to solvent complexes. The DMSO can be removed by vacuum evaporation, or an ALLTECH C18 disposable column can be used to exchange the solvent to a more volatile one and partially purify the products. The compounds are analyzed and purified by ternary gradient HPLC reverse phase chromatography. In all of the

reactions described below, the product was the major band in TLC, with a different mobility from the reactants under either butanol:ammonia or hexane:ethylacetate conditions. The compound numbers refer to the specific design. Every compound designed is given a unique name.

RWH10 Two equivalents of *para*-amino benzamidine HCL (0.416g) and 1 equivalent of αα'dibromomethyl *meta*-xylene (0.263g) were dissolved in 15ml of DMSO with stirring. The solution turns a light yellow, and after heating for 1 hour near the boiling point of DMSO turns a deep amber. The DMSO was removed via vacuum evaporation resulting in an amber colored oil. The oil is a single band on silica gel TLC. Recrystallization attempts by dissolving in water and/or methanol resulted in the same oil.

RWH23 Two equivalents of *para*-amino benzamidine HCL (0.416g) and 1 equivalent of 1,4 dibromo-2,3 butane diol (0.247g) were dissolved in 15ml of DMSO with stirring. A deep brown solution results fter heating for 1 hour near the DMSO boiling point. The DMSO was removed via vacuum evaporation resulting in a deep brown oil which was a single band on TLC. Recrystallization attempts by dissolving in water and/or methanol resulted in the same oil.

RWH26 Two equivalents of *para*-amino benzoic acid (0.274g) and 1 equivalent of αα'dibromomethyl *meta*-xylene (0.263g) were dissolved in 15ml of DMSO with stirring. The solution turns a light yellow, and after heating for 1 hour near the boiling point of DMSO turns a deep amber. Addition of water resulted in the precipitation of the product. The product was collected by filtration and recrystallized from methanol.

RWH28 Two equivalents of *para*-amino benzamidine HCL (0.416g) and 1 equivalent of αα'dibromomethyl *para*-xylene (0.263 g) were

dissolved in 15ml of DMSO. The αα'dibromomethyl *para*-xylene is less reactive than the *meta* compound because the amine formed in the first step of the reaction is further away from the other bromo-methyl group. Therefore, either extended heating or the addition of 0.25ml of tri-ethylamine as a catalyst is required for efficient reaction. The DMSO was removed by vacuum evaporation.

RWH29 Two equivalents of 2,6 diaminoanthraquinone (0.476g) and 1 equivalent of αα'dibromomethyl *meta*-xylene (0.263g) were dissolved in 15ml of DMSO with stirring. Because of the possibility of polymerization, the reaction was carried out slowly at room temperature over the course of 10 days. Then the reaction mix was briefly heated and the DMSO removed by vacuum evaporation. The material was recrystallized from methanol water solution.

RWH30 Two equivalents of *para*-amino benzamidine HCL (0.416g) and 1 equivalent of 2,6 αα'dibromomethyl pyridine (0.263g) were dissolved in 15ml of DMSO with stirring. The solution turns a light yellow, and after heating for 15 minutes near the boiling point of DMSO turns a dark brown. The reaction, once started, is exothermic and caution is necessary to avoid loss of the DMSO. The DMSO was removed via vacuum evaporation resulting in an brown colored oil. The oil is a single band on silica gel TLC. Recrystallization attempts by dissolving in water and/or methanol resulted in the same oil.

RWH32 Two equivalents of 2,6 diaminoanthraquinone (0.476g) and 1 equivalent of αα'dibromomethyl pyridine (0.263g) were dissolved in 15ml of DMSO with stirring. Because of the possibility of polymerization, the reaction was carried out slowly at room temperature over the course of 10days. Then the reaction mix was briefly heated and the DMSO removed

by vacuum evaporation. The material was recrystallized from methanol water solution.

RWH34 Two equivalents of 5-amino,8-hydroxy quinoline and 1 equivalent of αα'dibromomethyl *meta*-xylene were dissolved in DMSO with stirring. The solution was heated to initiate reaction, and the rapid reaction produced a brown oil. The compound was partially purified by bulk C18 column chromatography in a methanol/water system using an ALLTECH disposable column. The product elutes with the aqueous phase, as checked by TLC. The water and most of the DMSO were removed by vacuum evaporation.

RWH35 Two equivalents of 7-amino,4-hydroxy,2 naphthalene sulfonic acid and 1 equivalent of αα'dibromomethyl *meta*-xylene were dissolved in DMSO with stirring. The solution was heated to initiate reaction. The compound was partially purified by bulk C18 column chromatography in a methanol/water system using an ALLTECH disposable column. The product elutes with the aqueous phase, as checked by TLC. The product was then dried by air evaporation.

The Standard Assay for ASLV IN (1-286) and Core (52-207) Inhibitors

For ASLV IN and its catalytic core fragment, enzymatic assays were performed as in Bujacz et al. *J. Mol. Biol. 253:333-346,*(1995) with a duplex oligodeoxynucleotide substrate that represents the viral U3 DNA end described previously by Katz et al, *Annu. Rev. Biochem,* 63, 133-173 (1996). The substrate includes ^{32}P at the 5' end of the strand that will be cleaved in the processing reaction, and such processing is detected by a product that is 2 nucleotides shorter (-2) than the radioactively labeled strand. In the experiments reported here, the proteins were incubated at 30• C for 30 min. in the presence of various concentrations of inhibitor. The ^{32}P- labeled 18 base pair substrate was then added, and the reactions

incubated at 37• C, usually for 30 min. Final standard conditions were 2 uM ASLV IN or core fragment, 15 uM U_3 18/18 substrate , 0.1 uM to 1 mM inhibitor, 50 mM Hepes (pH 8.1), 50 mM NaCl, 2 mM 2-mercaptoethanol, 0.1% thiodiglycol, 10% DMSO, 4% glycerol, and 10 mM $MnCl_2$, in a 10 ul volume. Reactions were quenched by addition of EDTA, to 25 mM, and the products separated on 20% sequencing gels. Gels were analysed using a Fuji BAS1000 phospho-imaging system. For calculation of the extent of processing by IN, radiactivity in the major band at the –2 position was compared to the total in each reaction. To determine the effect of inhibitors, this value was compared to the control reaction that included no inhibitor. For calculation of the endonuclease activity of the catalytic core fragment of ASLV IN, radioactivity in a band at the –3 position, representing a preferred product of the core endonuclease, was compared to the total in each reaction.

For HIV-1 IN, assays were performed as in Assante-Appiah and Skalka, *Antiviral res.* 36, 139-152 (1997), with a duplex oligodeoxynucleotide substrate that represents the viral U5 DNA end described by Kulkowsky et al. *Vir.* 206:448 (1995). The design of the assay and reaction protocol were as described for ASLV IN. Final standard conditions were 1 uM HIV-1 IN, 0.1 uM U5 DNA 21/21 substrate, an alternative viral insertion site for HIV, in 20 mM Hepes (pH 7.5), 50 mM KCl, 5 mM DTT,10% glycerol, 6% DMSO, 10 mM $MnCl_2$. Incubation was at 37• C for 60 min. in a 10 ul volume.

Order-of-addition experiments were performed by incubating either the HIV enzyme with the inhibitor, or the enzyme with the DNA substrate (U5), or the DNA substrate with the inhibitor for 30 minutes at 30°C and then adding the other component (either DNA, inhibitor or enzyme) followed by the manganese. The reactions were then performed and analyzed as described above. For determination of the nature of inhibition, conditions were chosen in which the rate of processing was in the linear

range and the concentrations of inhibitor and substrate were varied as indicated.

Fluorescence Assay

The binding of compound RWH35 to integrase was assayed with a Jasco FP-750 spectrofluorimeter. The ASLV integrase and its isolated catalytic core fragment were stored in 50 mM HEPES, pH 8.1, 0.5 M NaCl, 1% thiodiglycerol, 0.1 mM EDTA and 40% glycerol. The RWH35 inhibitor was dissolved in water to 6 μM concentration. All assays were performed at 25°C. Two assays were performed. In one the concentration of the inhibitor was varied and in the other the concentration of the enzyme was varied. The two assays produce consistent results, but the binding constants are reported from the variation in the concentration of the inhibitor.

For ASLV integrase, the assay was run with varying concentrations of inhibitor and 0.296 μM of integrase or 0.55 μM of its core fragment. In the second assay, where the protein concentration was varied, the concentration of the inhibitor was 1.38 μM inhibitor for the intact integrase, and 1.25 μM inhibitor for the core fragment. With HIV, the assay varying the inhibitor concentration used 0.32μM integrase. Then the integrase concentration was varied in the presence of 1.25μM inhibitor.

The intrinsic fluorescence of integrase was measured using the protein tryptophan excitation at 230 nm. An emission peak at 345 nm for ASLV integrase and core, and 337 nm for HIV integrase was measured. The RWH35 compound fluoresces with excitation at 230 nm and peak emission at 420 nm. No fluorescence energy transfer was observed. The baseline was subtracted from all measurements. The change in fluorescence ΔF was plotted against ΔF/[RWH35] to obtain the apparent dissociation constant K_{app}.

Inhibitor effect on HIV integration in cell culture.

The expression of a lacZ reporter gene was used to assess viral integration in the presence of varying amounts of inhibitor. $5x10^4$ HeLa cells were plated onto each well of a 24-well plate. Two wells were analyzed for each inhibitor concentration. On the following day, a HIV-based retroviral vector which contained a lacZ gene (Naldinin et al. *Science*, 272: 263-267, 1996) was added to each well for 2 hrs in the presence of inhibitor and 5 mg/ml dextran. After 2 hrs, the HIV vector was removed and fresh medium containing the inhibitor was added. Inhibitor was kept on cells for another 16 hrs, then removed and fresh medium added. Cells were stained 2 days post infection using a β-gal assay performed according to the Transfection MBS mammalian transfection kit (Stratagene).

Inhibitor effect on ASLV integration in cell culture.

The expression of a drug resistant (neo^r) reporter gene was used to assess viral integration in the presence of varying amounts of inhibitor. $1x10^5$ HeLa cells were plated onto each 60 mm dish, and 2 dishes were analyzed for each inhibitor concentration. On the following day 1 ml of an ASLV IN+ vector which contained the neo^r reporter gene (Daniel et al. *Science* 284: 644-647, (1999) was added to each dish at 1:1000 dilution, together with the inhibitor and 10 mg/ml dextran. After 2 hrs the vector was removed and fresh medium added, containing the inhibitor. On the next day the inhibitor-containing medium was replaced with fresh inhibitor-free medium. The drug G418 was then added to a final concentration of 1 mg/ml. Surviving (neo^r) colonies were counted 6 days later.

Inhibitor effect on growth of HeLa cells.

$5x10^4$ HeLa cells were plated onto each well of a 24-well plate, 2 wells were analyzed for each inhibitor concentration. Cell numbers were

counted 3 days post plating. HeLa cells were carried in DMEM media supplemented with 10% fetal bovine serum and Penicillin/Streptomycin.

Molecular modeling

The complexes of RWH10 and RWH35 with the catalytic core of HIV integrase were modeled from the crystal structure of the 5CITEP complex with HIV integrase core (Goldgur et al., *Proc. Natl. Acad. Sci. U.S.A* 96, 13040-13043, 1999), in order to analyze possible modes of action. The program AMMP (Harrison, *J. Math. Chem.,* 26,125-137, 1993) was used with the current all-atom sp4 potential set (Weber and Harrison, *Protein Science,* 6, 2365-2374 (1997). The charge generation parameters were taken from Bagossi et al. *J. Mol. Biol. Model. 5*, 143-152 (1999). The models were generated by superimposing the base analog groups (napthylene in RWH35, and benzamidine in RWH10) on the similar group in 5CITEP. Because the active site in the crystal structure is near a crystallographic twofold axis and our compounds are symmetric both the 2:1 and 1:1 complexes were modeled. The coordinates were built using the Kohonen and analytic model building features of AMMP (Harrison, *J. Math. Chem.,* 26,125-137, 1993), and minimized with conjugate gradients. The amortized fast multipole algorithm in AMMP was used for the long range terms in the non-bonded and electrostatic potentials so that no-cutoff radius was used.

Results and Discussion

Rational for Selection of Chemical Groups in the Set of Related Compounds

These compounds were initially checked for anti-integrase activity because a related compound, pentamidine, is a topoisomerase inhibitor and topoisomerases catalyze strand transfer reactions, reactions that are not dissimilar to those of integrases. HIV assays were performed as described in Assante-Appiah & Skalka, *Antiviral Res.* 36, 139-152, (1997), the disclosure being incorporated herein by reference, with a substrate

that represents the viral U5 DNA end (Kulkowsky et al., *Vir.*, 206:448, 1995).

RWH10 is a conformationally restricted pentamidine analog and RWH23 is a pentamidine analog with a hydrophilic linker which mimics an intermediate in peptide bond hydrolysis. Since these molecules showed anti-integrase activity, a series of changes were designed to isolate important chemical features of the inhibitors. RWH26 and RWH29 tested the importance of the amidine groups by replacing them with an isostructural benzoic acid and an analog of a nucleic acid base. RWH28 and RWH30 further explored the geometry of the linker group by substituting a *para*-linker and a pyridine linker.

The activity results showed that the amidine group was not critical for activity. The linker results showed that a *meta*-linker was optimal, but the specific chemistry of the linker was not critical as long as it was a planar aromatic compound.

Structural similarities suggested that the end groups on the inhibitors may act as nucleic acid base analogs. In order to test this hypothesis, two additional compounds (RWH34 and RWH35) were prepared and tested. In these compounds 5-amino,8-hydroxy quinoline and 7-amino,4-hydroxy,2 naphthalene sulfonic acid represent base analogs. The fused ring aromatic system is predicted to stack well with nucleotide bases, and the hydrogen bonding groups mimic guanine nucleotides. None of this series of compounds are bis-catechols, but their general architecture is similar to the functional group-linker-aryl group arrangement described for another class of integrase inhibitors (Zhao et al., *J. Med. Chem.* 40, 242-249 1997).

Inhibition of ASLV and HIV-1 Integrases

Table I (*supra*) presents a summary of the IC_{60}s calculated for all of the newly synthesized compounds on the processing activities of HIV-1 and ASLV integrase, and the endonuclease activity of the ASLV integrase catalytic core fragment. Compounds RWH23, 26 and 28 proved to be

relatively poor inhibitors of all of three proteins, with IC_{50} values of 30-50 μM for HIV and 90-1000 μM for ASLV integrase or its core fragment. At mM concentrations pentamidine is also a very poor inhibitor of ASLV integrase.

Compounds RWH10, 29, 30 and 32 were moderately active inhibitors with IC_{50} values of 12-26 μM for HIV and about 100-360 μM for ASLV integrase. RWH30 showed unexpectedly poor inhibition of the ASLV integrase core fragment with an IC_{50} of 500 μM, compared to 220 μM for the full length protein.

The most potent inhibitors of HIV-1 integrase were RWH34 and RWH35, with submicromolar IC_{50} values of 0.7 and 0.8 μM, respectively. RWH34 and RWH35 were also the best inhibitors of the ASLV proteins, with IC_{50} values of 4-13 μM for the full length protein and its core fragment. Comparison of results with the three proteins shows that these compounds are more effective inhibitors of HIV-1 integrase than of ASLV, although RWH34 and 35 are the best inhibitors of both enzymes. This similarity implies that the compounds bind to a conserved region of the two integrase proteins and affect the reactions by a similar mechanism. Inhibition of the activity of the ASLV integrase catalytic core fragment implies that the inhibitor binds at or close to the active site.

The Inhibitors Act by Binding Directly to the Integrase

Both ASLV integrase and HIV integrase show an intrinsic fluorescence signal from Trp residues. Therefore, a fluorescence assay was used to obtain apparent binding constants for inhibitor and integrase. The fluorescence of HIV integrase decreases sharply with increasing concentration of the RWH35 inhibitor (**Figure 2A**). Calculation of the apparent dissociation constant of RWH35 for HIV-1 integrase is shown in **Figure 2B**. The apparent binding constants are: 21 nM for HIV-1 integrase, 14 nM for ASLV integrase and 120 nM for ASLV integrase core. As no magnesium, manganese, or DNA was present in these analyses, the

results indicate that the inhibitor binds directly to the integrase proteins. The apparent dissociation constants are very similar for the HIV-1 and ASLV integrase, although the IC_{50} values for inhibition of the reaction differ by an order of magnitude. The ASLV catalytic core fragment apparently binds the inhibitor less well than the full length integrase, although its activity was inhibited to about the same extent.

Enzyme Inhibition Kinetics

The kinetics of enzyme inhibition were studied for the most active compounds, RWH34 and RWH35. The inhibition showed no dependence on the order of addition (**Figure 3C**). This indicates that the compounds are acting as reversible inhibitors and not sequestering either enzyme or DNA substrate. These results indicate that the inhibitors are in kinetic equilibrium during the time scale of the experiment.

Figures 3A and **3B** show plots of 1/V vs. 1/[S] with no inhibitor and increasing concentrations of RWH34 and RWH35. These results indicate that these compounds are non-competitive inhibitors. This implies that there is an enzyme-inhibitor-substrate complex in equilibrium with enzyme inhibitor and enzyme substrate complexes. One interpretation of this result is that the inhibitors are binding in a site distal to or distinct from the DNA binding site, as is the case with some ASLV inhibitors (Wlodawer, A. and Vondrasek, J., *Annu. Rev. Biophys. Biomol. Struct.* 27: 249-284, 1998). The other interpretation is that the inhibitors are binding to a subsite or part of the active site. In either case they are not preventing the binding of the DNA to the integrase, but are preventing the processing reaction. Non-competitive inhibition of processing excludes a mechanism where the inhibitors simply mimic a blunt end DNA molecule, but it does not exclude the possibility that the inhibitors mimic nucleotides or bases.

Inhibition of HIV-1 integration in a tissue culture assay

The inhibitor RWH35 was assayed for the ability to inhibit viral integration of DNA in cell culture. The cells were exposed to virus and inhibitor simultaneously for 2 hours and then to inhibitor only for 16 hours. The viral vectors are competent to integrate in cell culture but are not competent to replicate. The results showed that this inhibitor was able to block integration for both HIV and ASLV vectors with an IC_{50} of approximately 5μM against both HIV and ASLV. This is comparable to the IC_{50} values reported in Hazuda et. al. *Science,* 287:646-650 (2000) for di-ketone inhibitors. This IC_{50} value is well below the effective toxicity of the compounds, as judged by the effects on cell viability (**Figure 4**).

Implications for the Integrase Mechanism

The major complication of interpreting the results for integrase is the absence of a structure of an integrase-DNA complex. Outside of general reaction poisoning by sequestering the substrate or the catalytic metal ions, two general types of inhibitory mechanism are envisioned. In the first mechanism, the inhibitors bind to the protein. In the second mechanism, the inhibitors bind to a complex of the integrase with either viral DNA or viral and target (host) DNA. These two possible mechanisms for inhibition are distinguished by the results of the fluorescence assay. An inhibitor which binds to an integrase-DNA complex would not be expected to show significant binding to the integrase in the absence of DNA. The experimental results of the fluorescence assay showed that at least one inhibitor (RWH35) binds to integrase proteins in the absence of DNA or added metal ions. Therefore, these inhibitors act by the first mechanism, and bind directly to the protein. Similar apparent binding constants were observed with both HIV and ASLV integrase, implying that the mechanism of inhibition of RWH35 is similar for both enzymes.

The diverse nature of the functional groups in these inhibitors implies that they are not simply binding to the catalytic metal ions in

integrase. Furthermore, as the catalytic manganese ions are present in excess (10mM $MnCl_2$ vs. μM concentrations of inhibitor) in the assay, it is unlikely that the compounds are acting as metal chelators, removing the catalytic ions from solution. Similarly, the order of addition experiments shows that the enzyme-inhibitor complex is in kinetic equilibrium within the time scale of the experiment. Thus, the compounds are not working by covalently modifying the protein or DNA.

Pentamidine and related compounds bind to DNA (Edwards, et al., *Biochemistry 31, 7104-7109,* 1992) in the minor groove. This activity is at least partially due to the cationic nature of the amidine groups. One of the most effective compounds (RWH35) is anionic, and would be unlikely to bind in the minor groove of DNA, due to electrostatic effects. This implies that the best of these integrase inhibitors does not bind to the minor groove in DNA. It is also noteworthy that the weakly anionic derivative (RWH26) is not a particularly effective inhibitor. Therefore, either the sulfonate or the specific geometry of the anionic groups is important for the activity of the compounds. Since the relatively neutral compound RWH34 is nearly as effective as RWH35, this implies that the geometry of the compound is important.

The compounds showed variable activity with respect to intact and core ASLV integrase. While compounds that were more active against intact enzyme were generally more active against the core, there were exceptions like RWH28 and RWH29. In addition, RWH35 showed nearly ten-fold higher apparent affinity for the intact integrase than the core in the fluorescence assay (**Table II**). This implies that the binding site for the inhibitor is made of residues from the core and at least one terminal domain of the integrase, or that the inhibitor causes a conformational change that involves more than one domain.

Table II: Apparent Kd for RWH35 from changes in intrinsic fluorescence

Integrase	Kapp (nM)
HIV-1 IN	21
ASLV IN	14
ASLV Core	120

The molecular geometry of the compounds of the present invention are critical to their action. Pentamidine shares functional groups with the tested compounds (especially RWH10, RWH23, RWH28, RWH29, and RWH30), but is relatively inactive against the integrases. Therefore, the choice of the central linker is important. Molecules with flexible and hydroxylated linkers, as in RWH23, or simply flexible, as in pentamidine, tend to be less active than molecules with rigid and aromatic linkers. The internal geometry of the linker is also critical. RWH10 and RWH28 differ by being *meta* and *para* stereoisomers, and RWH10 is significantly more active than RWH28. On the other hand, RWH10 and RWH30 differ by replacing the central phenyl ring with a pyridine and have essentially the same activity, which shows that the geometry rather than specific chemistry of the central linker is important. Therefore, the results imply that the entire inhibitor molecule is involved in inhibition.

The recently determined crystal structure of HIV integrase with 5CITEP (Goldgur et al., *Proc. Natl. Acad. Sci. U.S.A* 96, 13040-13043, 1999), is used to aid in understanding the mechanism of inhibition of the compounds of the present invention. In this structure the inhibitor is bound in the active site adjacent to the catalytic metal ion. The integrase binding site for this inhibitor contains residues that interact with both a base analog group and an aromatic linker group, as occurs in the compounds of the present invention. This binding site is different from that seen in the ASLV integrase-inhibitor complex, where the binding site is distal to the active site (Bujacz, G., et al., *J. Biol. Chem.* 272: 18161-

18168, 1997). The alternative distal binding site contains fewer possible contacts for the linking group and is therefore a less likely starting point for modeling, as experiments presented herein imply that the structure of the linking groups is important. The proximal binding site is adjacent to a crystallographic two-fold axis. Because the compounds of the present invention are symmetric they might bind to two adjacent active sites in an integrase dimer. However, it is important to remember that this two-fold axis could also be an artifact of crystallization. Therefore, models were prepared for both the dimeric 2:1 and monomeric 1:1 complexes of integrase and inhibitor.

The base analog group, either the sulfono-napthylene in RWH35 or the benzamidine group in RWH10, was modeled by superimposing it on the napthyl moiety in 5CITEP. This superposition was performed with both 2:1 and 1:1 models of the complex, and produced identical models for the integrase-inhibitor interactions. The 4-hydroxy group in RWH35 is capable of entering the coordination shell of the catalytic ion. A similar interaction could occur in the bis-catechol compounds described in Zhao et al., *J. Med. Chem.* 40, 242-249 (1997). The sulfonate in RWH35 is in position to interact with histidine 67 and glutamate 98. The models of RWH10 cannot place the amidine nitrogens into the coordination shell of the metals, rather they are placed adjacent to two aspartic acids (64, 116) which chelate the metal ion. The amidine nitrogens interact with histidine 67 and glutamate 98, this interaction is less specific than predicted for RWH35, which is consistent with the higher IC_{50} for RWH10 compared to RWH35.

Unlike the base analog, the conformation of the linking group differs between the different model complexes. In models of the 2:1 complex, the linker sits near the crystallographic two-fold and the *meta*-geometry is important for placing the two base analog groups into the active sites. The *para*-geometry would not allow the formation of this complex, and the flexible linker used in RWH23 is too short to allow the formation of this complex. In the 1:1 complexes, the linker lies along the

surface of the protein and fills a pocket adjacent to isoleucine 151. Again the *meta*-geometry is optimal for filling this pocket, and both the *para*-geometry and flexible hydrophilic linkers would not complement the structure in this region. It is possible to place the other base analogs in several locations with the models of the 1:1 complexes. However, it is difficult to assess which of these other locations is more likely to occur. In the models of both the 2:1 and 1:1 complexes the atom which corresponds to the nitrogen in the pyridine ring in RWH30 and RWH32 does not interact directly with the protein. This is consistent with the observation that the pyridine substitution makes little difference in the activity of the compounds.

Conclusions

A series of integrase inhibitors was designed, synthesized and tested. The initial designs were pentamidine analogs, but unlike pentamidine they are active as integrase inhibitors. Computer modeling implies that they fit into the structurally conserved regions of ASLV and HIV integrases, and the predicted integrase-inhibitor interactions agree with the relative inhibition values. The best compound has an IC_{50} of 0.7μM for HIV integrase and 6μM for ASLV integrase. Fluorescence quenching studies imply that the apparent binding constants for the compounds are nanomolar and similar in magnitude to the binding constants for substrates (Yi et. al., *Biochemistry,* 38:8458-8468 (1999). The best compound is active in cell culture and inhibits the replication of both ASLV and HIV at concentrations below cell toxicity. Therefore, the novel integrase inhibitors of the present invention are potential lead compounds for the design of clinically effective anti-retroviral agents.

The present invention is a series of new compounds that inhibit some medically important enzymes, significantly the retroviral integrases. The similarities between the catalytic activity of topoisomerases and integrases also allow for these new compounds to be used to inhibit

topoisomerases, specifically the bis-benzamidine compounds RWH10, RWH23, RWH28 and RWH30 of the present invention. Topoisomerase inhibition is therapeutically beneficial in protozoan and fungal infections. A further use for this series of compounds, more particularly the bis-benzamidine compounds RWH10, RWH23, RWH28 and RWH30 of the present invention, is to inhibit the catalytic activity of skin proteases. Certain skin conditions, such as exfoliative conditions, are caused by deregulation of skin proteases (tryptases). Therefore, the new compounds of the present invention are also useful in treatment of these debilitating diseases.

Integrases are essential enzymes in the life cycle of retroviruses, HIV-1, the causative agent of AIDS, being one such virus. As there are currently no known inhibitors of this enzyme, and HIV rapidly develops resistance to drugs, there is a long felt need to develop new compounds that will serve as lead compounds for the development of efficacious anti-AIDS therapeutics.

CLAIMS:

What is claimed is:

1. An integrase inhibiting compound, comprising:

a) a linker group, comprising a rigid aromatic compound with at least one functional group replacing an atom of said rigid aromatic compound,

b) said functional group bonded to said linker group, comprising at least one derivitized mono or poly cyclic aromatic group, and

c) said bond between said functional group and said linker group, comprising a flexible linkage.

2. The compound of claim 1, further comprising a symmetric compound.

3. The compound of claim 1, further comprising an asymmetric compound.

4. The compound of claim 1, wherein said functional group further comprises mimicking nucleotides or bases of a DNA.

5. The compound of claim 4, wherein said nucleotides or bases comprise placement into a stable hydrogen bonding geometry and base stacking geometry on said integrase.

6. The compound of claim 1, wherein said functional group is selected from the group consisting of *para*-amino benzamidine; *para*-amino benzoic acid; 2,6

diaminoanthraquinone; 5-amino, 8-hydroxy quinoline; and 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid.

7. The compound of claim 1, wherein said rigid aromatic compound is a planar rigid aromatic compound.

8. The compound of claim 1, wherein said linker group is selected from the group consisting of *meta*-xylene, *para*-xylene, and 2,6- dimethyl pyridine.

9. An integrase inhibiting compound, comprising:

a) a linker group, comprising a rigid non-aromatic compound with at least one functional group replacing an atom of said rigid non-aromatic compound,

b) said functional group bonded to said linker group, comprising at least one derivitized mono or poly cyclic aromatic group, and

c) said bond between said functional group and said linker group, comprising a flexible linkage.

10. The compound of claim 7, further comprising a symmetric compounds.

11. The compound of claim 7, further comprising an asymmetric compound.

12. The compound of claim 7, wherein said functional group is selected from the group consisting of *para*-amino benzamidine; *para*-amino benzoic acid; 2,6 diaminoanthraquinone; 5-amino, 8-hydroxy quinoline; and 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid.

13. The compound of claim 7, wherein said linker group is 1,4-diamino 2,3-butanediol.

14. A method of inhibiting an integrase, comprising binding of an integrase inhibiting compound, comprising:

a) a linker group, comprising a rigid aromatic compound with at least one functional group replacing an atom of said rigid aromatic compound,

b) said functional group bonded to said linker group, comprising at least one derivitized aromatic phenyl naphthyl group, and

c) said bond between said functional group and said linker group, comprising a flexible linkage;

to an integrase, inhibiting a catalytic activity of said integrase and inhibiting an essential function in viral replication of said retrovirus.

15. The method of inhibiting an integrase of claim 14, wherein said integrase inhibiting compound binds at or close to the active site on said integrase.

16. The method of inhibiting an integrase of claim 14, wherein said integrase inhibiting compound binds in a site distal to or distinct from the DNA binding site on said integrase.

17. The method of inhibiting an integrase of claim 14, wherein said integrase inhibiting compound binds directly to a viral DNA-integrase complex.

18. The method of inhibiting an integrase of claim 14, wherein said integrase inhibiting compound binds directly to a viral and host DNA-integrase complex.

19. The method of inhibiting an integrase of claim 14, wherein said integrase inhibiting compound inhibits said integrase by inhibiting a viral integration a viral DNA into a host DNA.

20. The method of inhibiting an integrase of claim 19, further comprising an inhibition of a processing step of an integration of said viral DNA into said host DNA.

21. An integrase inhibiting compound, comprising:

a) a linker group, comprising a rigid aromatic compound with at least one functional group replacing an atom of said rigid aromatic compound,

b) said functional group bonded to said linker group, comprising at least one derivitized mono or poly cyclic aromatic group, and

c) said bond between said functional group and said linker group, comprising a flexible linkage,

wherein said integrase inhibiting compound is a lead compound for further development of a therapeutic agent that causes inhibition of an integrase.

22. The integrase inhibiting compound of claim 21, wherein said inhibition of an integrase further comprises inhibition of replication of a virus at concentrations below cell toxicity.

23. An integrase inhibiting compound, comprising a mixture of 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid and a compound comprising a diamino methyl meta-xylene linker group with two functional groups comprising 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid.

24. An integrase inhibiting compound comprising 8-bromo, 7-amino, 4-hydroxy, 2 naphthalene sulfonic acid.

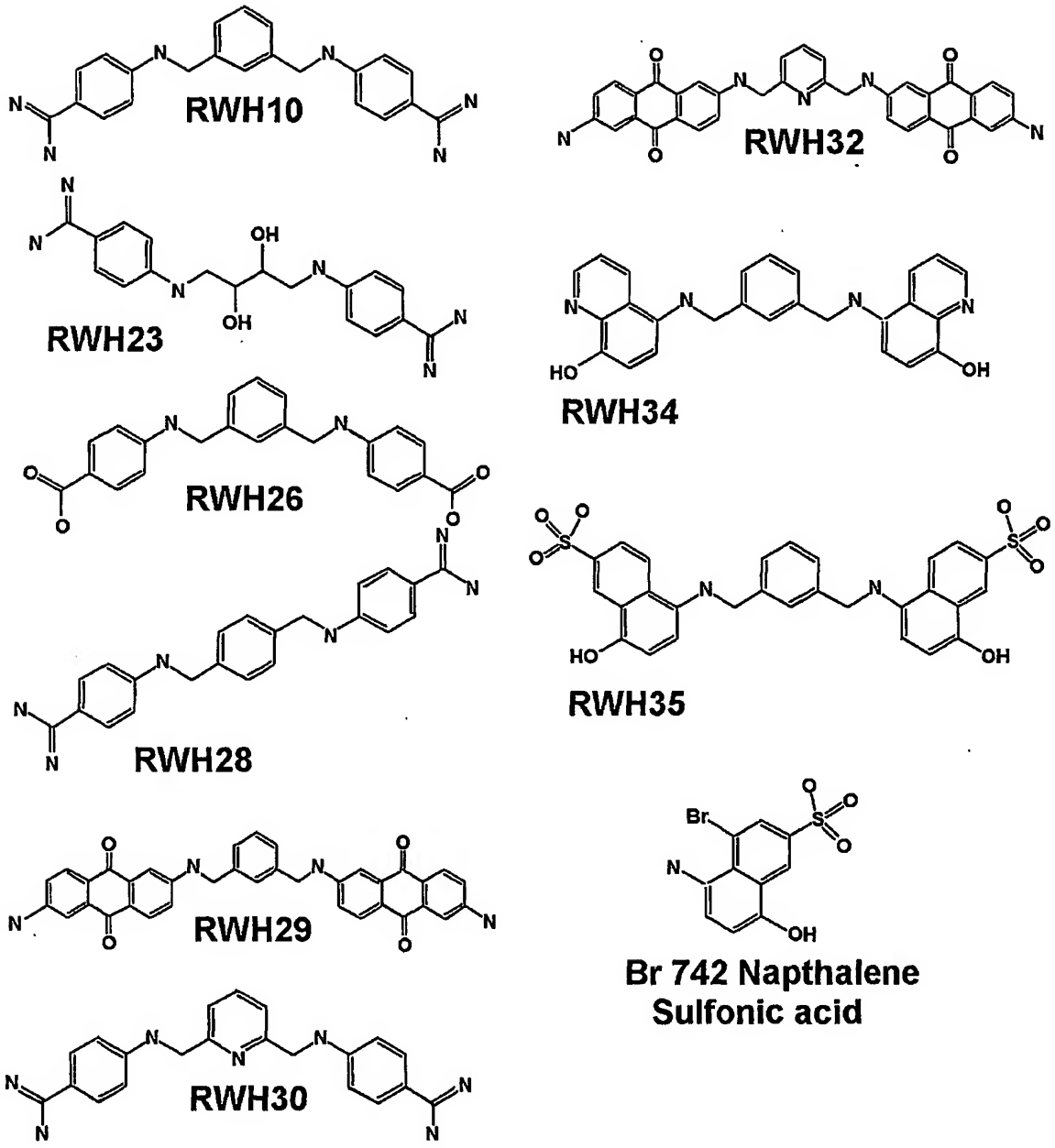
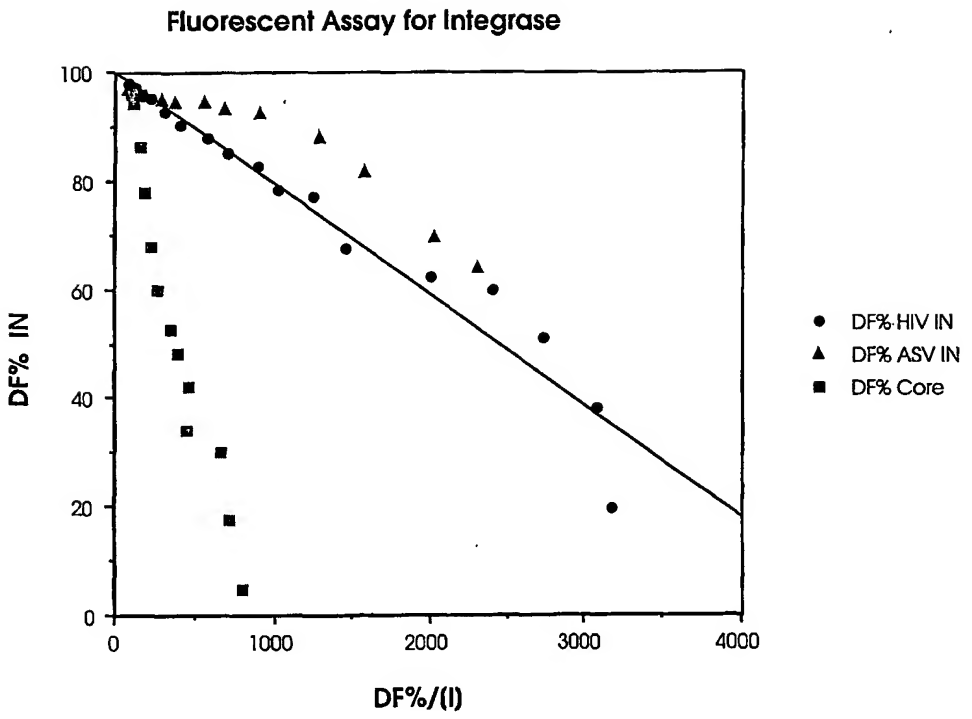

RWH10
RWH32
RWH23
RWH34
RWH26
RWH35
RWH28
RWH29
Br 742 Napthalene
Sulfonic acid
RWH30

Fig. 1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DF%/(I)
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0
1000
2000
3000
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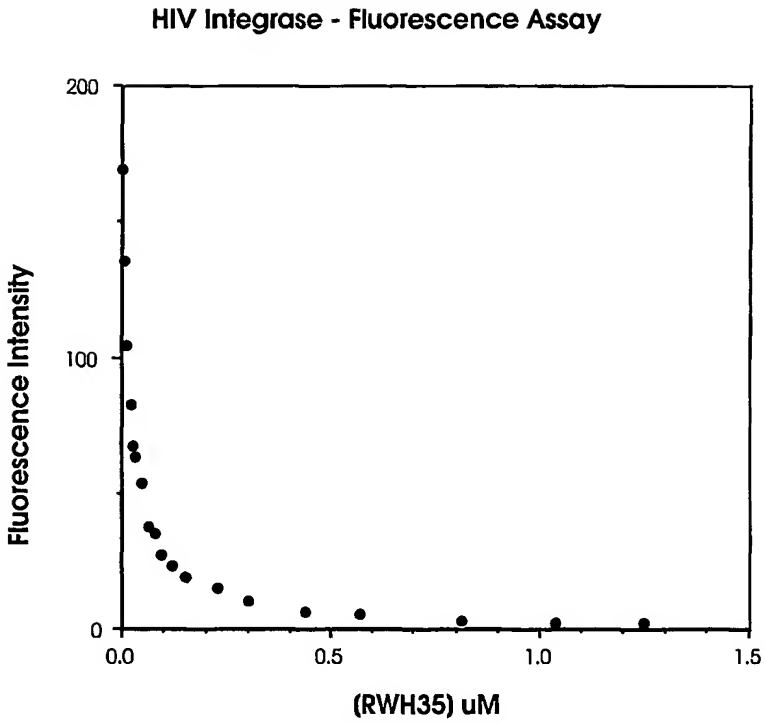
DF% HIV IN
DF% ASV IN
DF% Core

Fig. 2a


HIV Integrase - Fluorescence Assay
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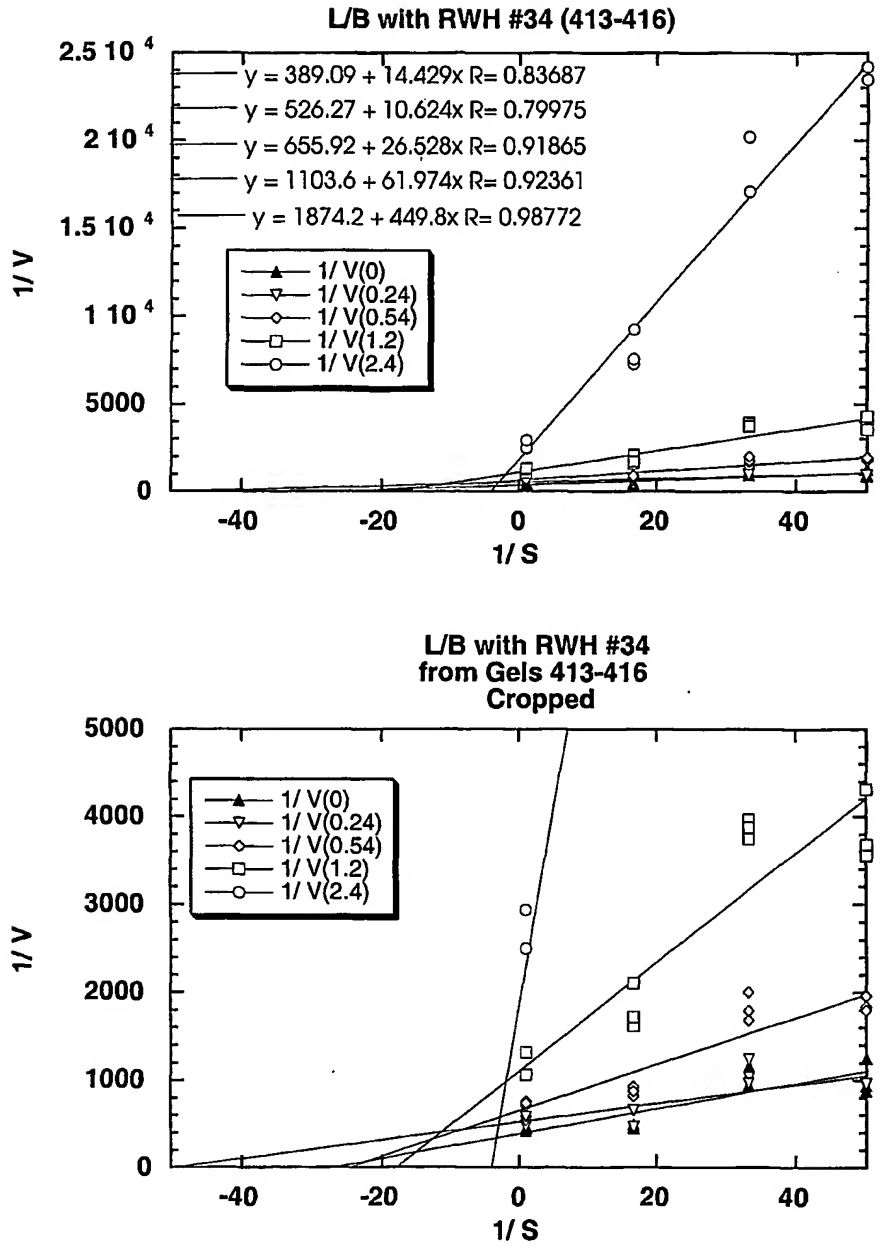
1.5
(RWH35) uM

Fig. 2b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y = 526.27 + 10.624x R= 0.79975
y = 655.92 + 26.528x R= 0.91865
y = 1103.6 + 61.974x R= 0.92361
y = 1874.2 + 449.8x R= 0.98772
1/ V(0)
1/ V(0.24)
1/ V(0.54)
1/ V(1.2)
1/ V(2.4)
2.5 10 4
2 10 4
1.5 10 4
1 10 4
5000
0
1/ V
-40
-20
0
20
40
1/ S
L/B with RWH #34
from Gels 413-416
Cropped
5000
4000
3000
2000
1000
0
1/ V
1/ V(0)
1/ V(0.24)
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1/ V(2.4)
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-20
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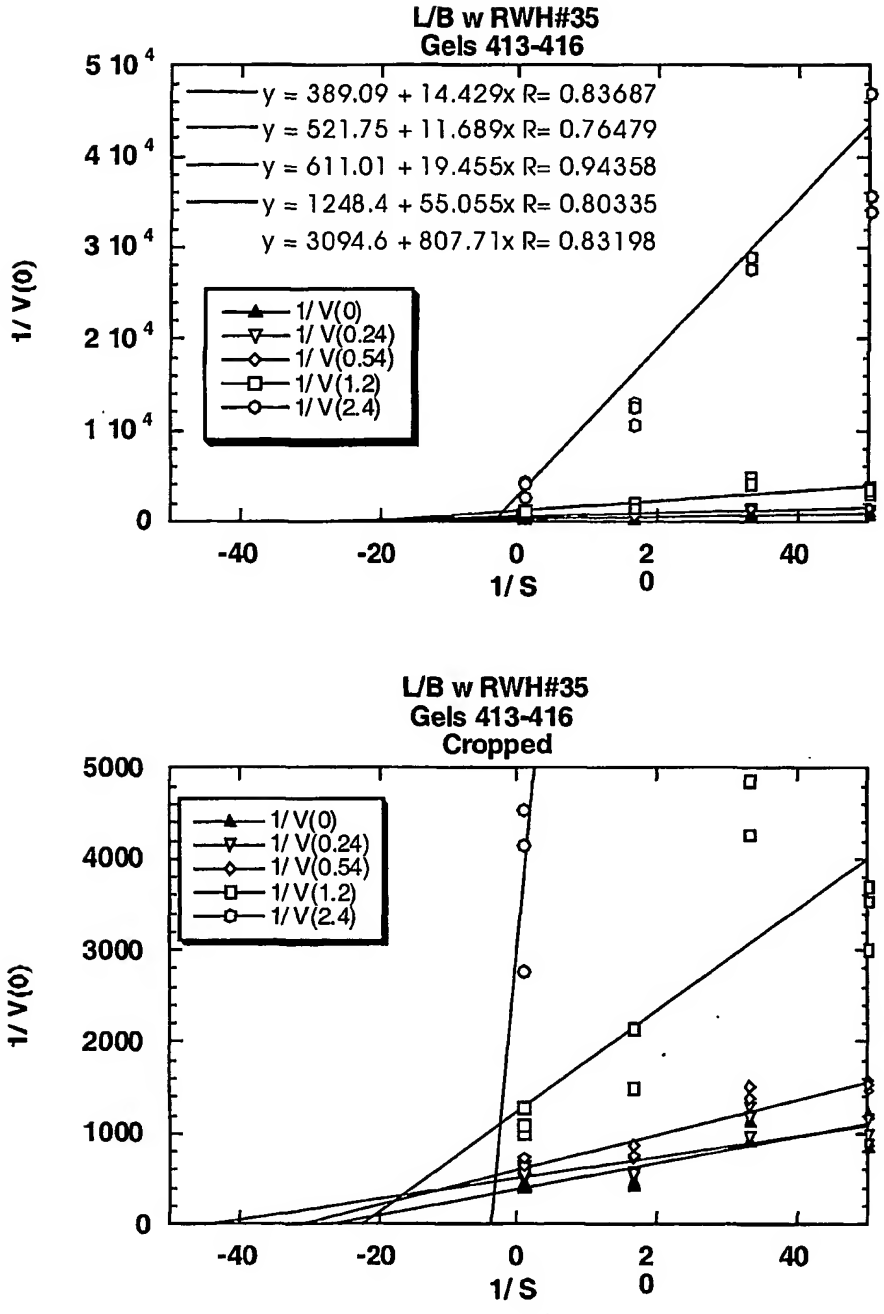
20
40
1/ S

Fig. 3a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Gels 413-416
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y = 611.01 + 19.455x R= 0.94358
y = 1248.4 + 55.055x R= 0.80335
y = 3094.6 + 807.71x R= 0.83198
1/ V(0)
1/ V(0.24)
1/ V(0.54)
1/ V(1.2)
1/ V(2.4)
1/ V(0)
5 10 4
4 10 4
3 10 4
2 10 4
1 10 4
0
-40
-20
0
2
0
40
1/ S
L/B w RWH#35
Gels 413-416
Cropped
1/ V(0)
1/ V(0.24)
1/ V(0.54)
1/ V(1.2)
1/ V(2.4)
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-40
-20
0
2
0
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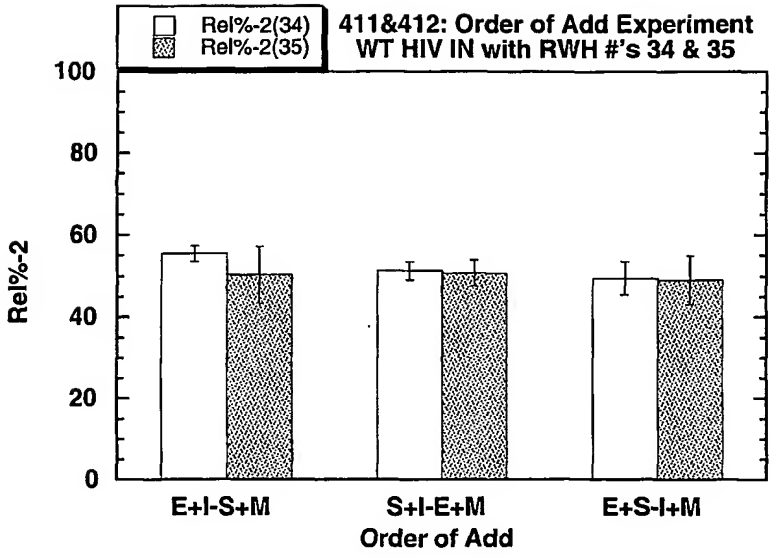
1/ S

Fig. 3b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Rel%-2(35)
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S+I-E+M
E+S-I+M
Order of Add

Fig. 3c

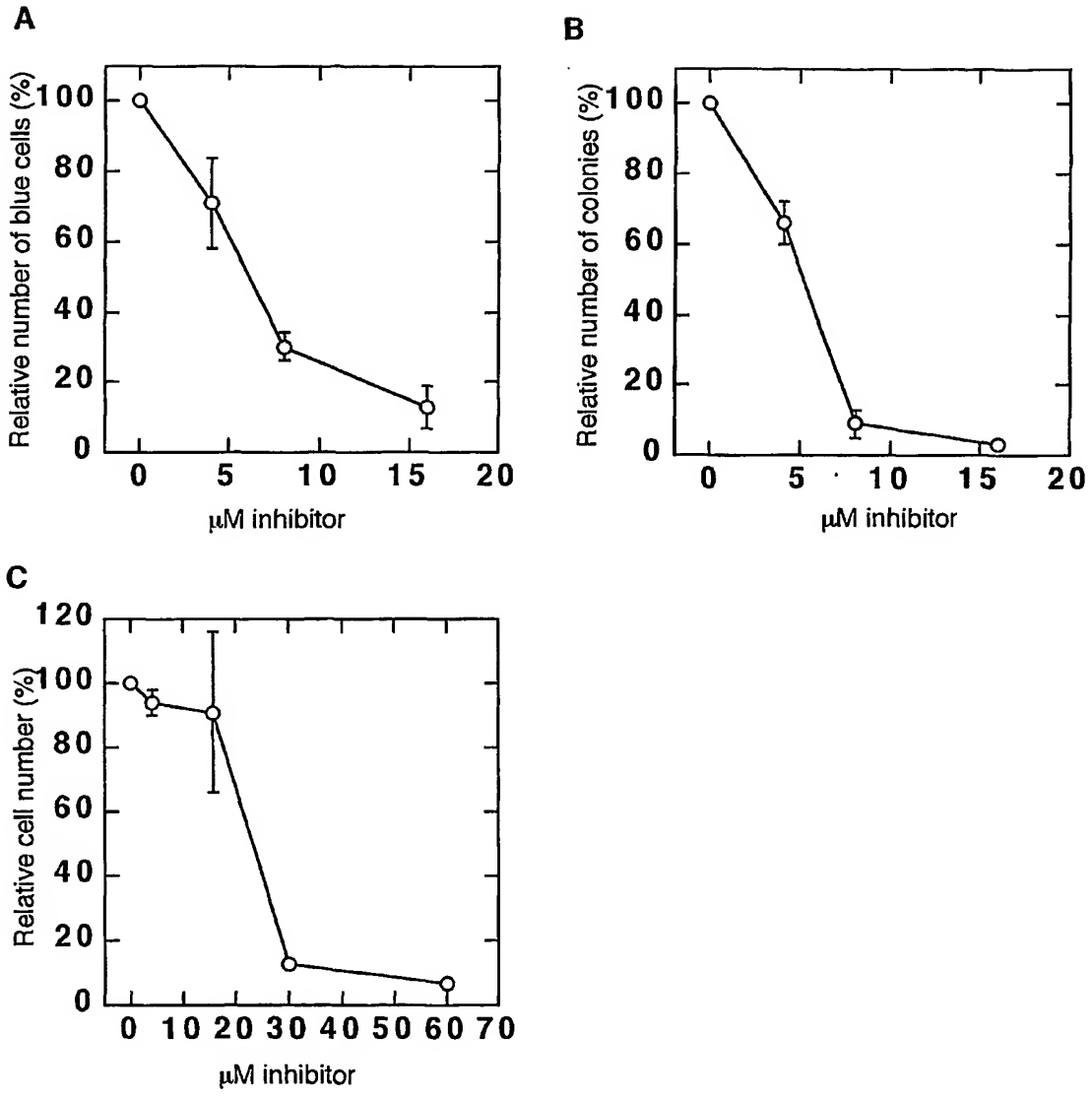
A
Relative number of blue cells (%)
100
80
60
40
20
0
0 5 10 15 20
μM inhibitor
B
Relative number of colonies (%)
100
80
60
40
20
0
0 5 10 15 20
μM inhibitor
C
Relative cell number (%)
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70
μM inhibitor

Fig. 4

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60/215,474 30 June 2000 (30.06.2000) US

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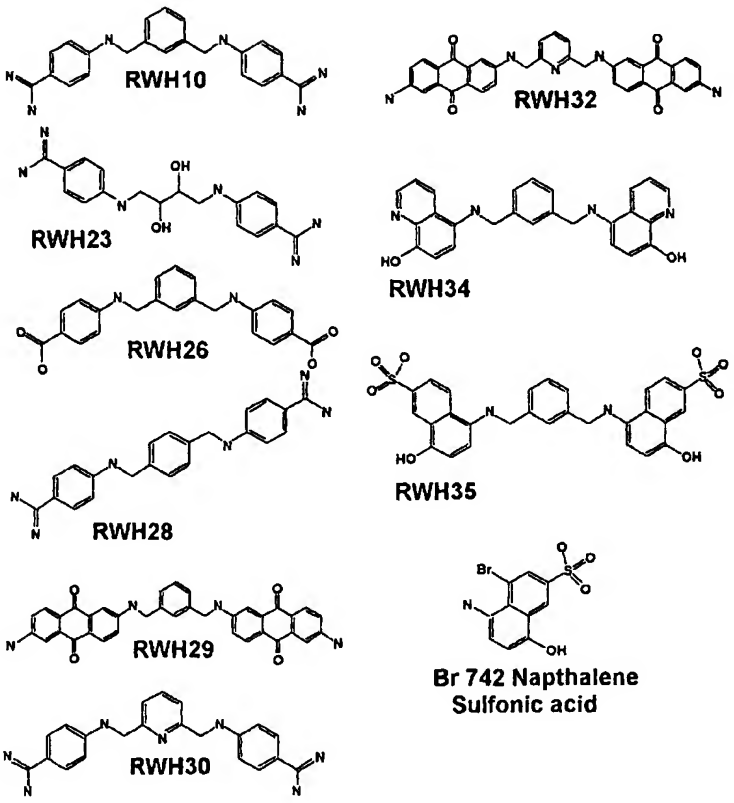
(54) Title: INHIBITORS OF HIV INTEGRASE



(57) Abstract: The present invention is a series of novel and effective inhibitors of integrase, an essential in the life cycle of retroviruses. These compounds were designed to have a restricted conformation for the determination of the integrase binding site and mechanism of inhibition. The integrase inhibitors of the present invention are effective in the submicromolar range, and thereby provide novel lead compounds for the development of anti-viral therapeutics.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07C257/18 C07C229/60 C07C225/34 C07C309/50 C07D213/38
C07D215/38 A61K31/135 A61K31/155 A61K31/185 A61K31/195
A61K31/44 A61K31/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HE ZHAO ET AL: "COUMARIN BASED INHIBITORS OF HIV INTEGRASE" JOURNAL OF MEDICINAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. WASHINGTON, US, vol. 40, no. 2, 1997, pages 242-249, XP002092069 ISSN: 0022-2623 cited in the application page 242 -page 244 ---	1-5,7,8, 14-22
X	HE ZHAO ET AL.: J. MED. CHEM., vol. 40, 1997, pages 1186-1194, XP000926749 page 1186 -page 1190 ---	1,3-5,7, 14-22
X	WO 99 48371 A (UNIV CALIFORNIA (US)) 30 September 1999 (1999-09-30) claim 15; figure 3B --- -/--	1,3-5,7, 14-22

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 January 2002	07.05.02

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Krische, D

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 50347 A (UNIV CALIFORNIA) 12 November 1998 (1998-11-12) abstract; claims 1-3; example 7 tab.1: cpds.117-130, 160-183	1,2,4,5, 7,14-22
X	KING, P. J. ET AL: "STRUCTURE-ACTIVITY RELATIONSHIPS: ANALOGUES OF THE DICAFFEOYLQUINICAND DICAFFEOYLTARTARIC ACIDS AS POTEN INHIBITORS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INTEGRASE AND REPLICATION" JOURNAL OF MEDICINAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY. WASHINGTON, US, vol. 42, no. 3, 1999, pages 497-509, XP000919203 ISSN: 0022-2623 cited in the application the whole document	1,2,4,5, 7,14-22
X	WO 98 38170 A (UNIV NORTH CAROLINA) 3 September 1998 (1998-09-03) claim 1 page 26 -page 28	1,2,4,5, 7,21,22
X	WO 99 25327 A (WARNER LAMBERT CO (US)) 27 May 1999 (1999-05-27) abstract page 28	1,2,4,5, 7,8
X	DE 19 31 122 A (FUJI PHOTO FILM CO.) 12 February 1970 (1970-02-12) claim 1 page 7, paragraph 2 - paragraph 3	1,2
X	EP 0 672 727 A (CIBA GEIGY AG) 20 September 1995 (1995-09-20) example 5	1,2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/19923

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-8, 14-23

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-8,14-23

Integrase inhibiting compound, comprising a rigid aromatic linker group with at least one aromatic functional group bound thereto and method of inhibiting an integrase using this compound.

2. Claims: 9-13

Integrase inhibiting compound, comprising a rigid non-aromatic linker group with at least one aromatic functional group bound thereto.

3. Claim : 24

Integrase inhibiting compound, comprising 8-bromo-7-amino-4-hydroxy-2-naphthalene sulfonic acid.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-8,14-22 relate to an extremely large number of possible compounds and methods using these. The function of inhibiting integrase is to be regarded as a desideratum and not as limiting characteristic. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds/methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds of fig.1 and closely related homologous compounds and methods using these.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/19923

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9948371 A	30-09-1999	AU 3366899 A	18-10-1999
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